# Studies on Proteolytic, Fibrinolytic, Antitryptic and Antifibrinolytic Activities in the Blood of Rats. (18370)

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The possible importance of the equilibrium between plasmin* ("fibrinolysin") and its inhibition in relation to clot lysis, blood coagulation, protein breakdown, and the body's response to various stimuli has been recognized only recently(1,2). It has been established that various types of stresses will cause an increased breakdown of protein. The nature of the mechanism of this physiological process is at present little understood.

This paper describes certain experiments, designed to contribute to an understanding of the sequences of increased protein catabolism observed under various conditions of stress.

*Experiments and results.* Male Wistar rats, weighing 215 to 330 g and fed on Purina dog chow checkers, were used. The animals were fasted and water was withheld for 24 hours before the experiment. The procedure for shock production was the same as previously described(3,4). Elastic rubber bands (Eberhard Faber No. 30) were applied to both hind limbs for 4½ hours and the blood drawn by heart-puncture 2 to 11½ hours after tourniquet release. It was found that the conditions described yielded a 24 hour mortality of 96%.

A. *Proteolytic and antitryptic activity of blood of rats in "tourniquet shock."* The proteolytic activity (pH 7.3) of the serum of normal control rats, determined according to Downie and Cliffton(5), was found to be practically zero, and no increase could be demonstrated in the serum of rats in "tourniquet shock" (total of 47 animals; blood drawn 2 to 16 hours after release of the tourniquets). Chloroform activation(1) did not reveal either any differences in the proteolytic activity of the serum of normal and shocked rats. The activity after chloroform treatment (25°, 16 hr) corresponded to about 8γ of trypsin per ml of serum in both groups (total of 83 animals). The antitryptic activity, determined essentially according to Tallan, Cliffton and Downie (6), was found to be slightly, but not significantly, lower than in the serum of normal rats (total of 84 animals).

There was the possibility that the tests employed in measuring proteolytic and antitryptic activity were not sufficiently sensitive to detect small changes in the enzymatic activity of serum. It was, therefore, investigated whether the measurement of the fibrinolytic and antifibrinolytic activity might be a more sensitive, possibly significant indicator during conditions of stress.

B. *Action of various proteolytic enzymes on the degree of clotting ability and total hydrolysis of fibrinogen.* No data seem to be available comparing the action of proteolytic enzymes on the clotting ability of fibrinogen and the hydrolysis to non-protein compounds. Fibrinogen, therefore, was incubated with the enzyme preparations listed in Table IB. The decrease in the clotting ability of fibrinogen was followed simultaneously with an increase in the amount of non-protein tyrosine. The extent of the loss of the clotting ability was determined by measuring up to what dilution the fibrin clot was detectable, or what con-

* This term is used throughout, following the suggestion made in the Transactions of the First Conference "Blood Clotting and Allied Problems," Josiah Macy, Jr. Foundation. p38, New York City, 1948.

1. MacFarlane, R. G., and Biggs, R., *Blood*, 1948, v3, 1167.

2. Mole, R. H., *J. Path. Bact.*, 1948, v60, 413.

3. Hamolsky, M. W., Gierlach, Z. S., and Jensen, H., *Am. J. Physiol.*, in press.

4. Gray, J. L., Botkin, A. L., Moulden, E. J., and Jensen, H., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 189.

5. Downie, G. R., and Cliffton, E. E., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 138.

6. Tallan, H. H., Cliffton, E. E., and Downie, G. R., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 667.

TABLE I. Effect of Various Proteolytic Enzymes On Clotting Ability and Total Hydrolysis, In % of Total Fibrinogen.

A. *Fibrinogen conc. and clotting time*
All sol. in veronal pH 7.35.
*Fibrinogen sol.* = 20 mg fibrinogen, containing 40-50% citrate, in 6 ml veronal.
*Clotting time* = 1 ml of diluted fibrinogen + 0.5 ml of 0.5% hemostatic globulin.

| mg Fibrinogen per 1.5 ml of final reaction mixture | .83 | .42 | .21 | .11 | .05 | .025 | .013 |
|---|---|---|---|---|---|---|---|
| Clotting time in sec (avg of 9 exp.) | 13±2.9 | 16±2.8 | 30±5.2 | 58±6.5 | 160±35 | ‡ | ‡ |

B. *Action of enzymes at 38°C.*

| Enzyme | Plasmin ("Fibrinolysin") | | Trypsin | | Chymotrypsin | | $CHCl_3$-activated serum |
|---|---|---|---|---|---|---|---|
| No. of exp. | 6 | 5 | 15 | 11 | 5 | 20 | 2 |
| Conc. of enzyme per ml of final digestion mixture | 670 γ = 0.2 U* | 670 γ = 0.2 U* | 1.7 to 3.3 γ | 3.3 to 6.7 γ | 12.5 to 16.7 γ | 8.3 to 16.7 γ | 0.17 ml |
| Minutes after start at 38°C | 3 | 5 to 8 | 2 to 5 | 5 to 10 | 3 | 5 to 15 | 30, 45 |
| % Destruction of clotting ability | 96 | >97 | 96 | >97 | 94 | >97 | 100† |
| % Hydrolysis to NP-compounds | 1.4 | 3.0 | 1.8 | 3.5 | 9.0 | 14.8 | 5.0 |

* U = units according to Loomis(8).
† Lysis of fibrin clot.
‡ No clot.

centration of fibrinogen corresponded to a certain clotting time (Table IA). The percentage of proteolysis was estimated by converting optical density to mg of tyrosine-tryptophane, having the same ratio as in fibrinogen (5.8% tyrosine, 3.3% tryptophane). The results obtained by the above methods although approximations, furnish sufficient information about the comparative sensitivity of these test procedures. 15.0 ml of 0.4% fibrinogen† in veronal buffer of pH 7.35(7) was mixed with 3.0 ml of the enzyme solution in the same buffer and incubated at 37.5°C. At the start of the experiment, and after certain time intervals, the remaining fibrinogen was clotted by adding 1.0 ml of the digestion mixture to 0.5 ml of a 0.5% solution of thrombin (hemostatic globulin‡) in veronal buffer. In the experiments with trypsin, the thrombin solution employed contained 10 γ of crystalline trypsin inhibitor (soybean) per ml to stop the enzymatic action during the process of clotting. In separate experiments, it was found that the addition of this amount of inhibitor did not influence the clotting time. Simultaneously, the amount of non-protein tyrosine-tryptophane was estimated colorimetrically(5).

Table I demonstrates that using various proteolytic enzyme preparations,§ more than 97% of the clotting ability of fibrinogen is destroyed when only a very small percentage is hydrolyzed to non-protein compounds.

These results indicate: 1. The disintegration of fibrinogen to non-clottable products is by far a more sensitive indicator than the proteolytic disintegration to non-protein compounds. 2. The very first attack of these enzymes on fibrinogen is directed towards those groups which are responsible for the process of clot formation.

C. *Comparative proteolytic effects of trypsin and plasmin on casein and fibrinogen.* The possible identity of the serum proteolytic enzyme ("serum tryptase") with the fibrinolytic-fibrinogenolytic enzyme "plasmin" is indicated by the above results. This assumption

† Preparation of bovine origin, containing 40-50% citrate, obtained from Armour Laboratories, Chicago, Ill.

7. Clark, D. G. C., Cliffton, E. E., and Newton, B. L., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 276.

‡ We wish to express our appreciation to Dr. H. D. Piersma of Lederle Laboratories, Pearl River, N. Y., for generously supplying us with this preparation.

§ We wish to express our appreciation to Dr. E. C. Loomis of Parke, Davis and Co., Detroit, Mich., for generously supplying us with various fibrinolysin preparations used in these studies.

is further strengthened by results of a preliminary comparative study of the proteolytic activity of trypsin and plasmin, under experimental conditions mentioned above, using casein and fibrinogen as a substrate. It was observed that the effect of 1 $\gamma$ of crystalline trypsin (approx. 0.2 fibrinolytic units) in splitting casein to non-protein compounds equalled that of about 200 $\gamma$ (0.06 fibrinolytic units)(8) of the "fibrinolysin" preparation employed. It should be emphasized that this result was obtained with a plasmin preparation, the absolute purity of which was not known. The same relation was found with fibrinogen as a substrate. The rate of degradation, produced by both enzymes, was about 4 to 5 times higher for casein than for fibrinogen as indicated by the amount of tyrosine-tryptophane liberated. About the same ratio was observed for chloroform-activated serum.

D. *Fibrinolytic and antifibrinolytic activity of blood of rats in "tourniquet shock" and after epinephrine administration.* On the basis of the above results, an attempt was made to use the destruction of the clotting ability of fibrinogen as a test method for fibrinolytic activity of blood of rats, which had been subjected to "tourniquet shock." However, this was found unsatisfactory, as it is very difficult and often impossible to avoid the formation of thrombin, and consequently clot formation in the blood from rats in "tourniquet shock" while the blood is being drawn into the syringe. Therefore, the method of MacFarlane and Pilling(9), which measures the dissolution time of a fibrin clot, was employed for the determination of fibrinolytic activity. However, this method allows only an arbitrary expression of activity, which in this case was in terms of the greatest dilution of the plasma that will produce lysis (within 24 hours at 37°C) of the clot formed in a 0.018% solution of fibrinogen. Antifibrinolytic activity was determined according to Guest, Daly, Ware and Seegers(10,11). Armour's bovine fibrinogen was used as substrate. The required amount of thrombin (hemostatic globulin, Lederle) was added in 0.1 ml of saline, giving a final reaction volume of 0.5 ml. The inactivation of plasmin by plasma was carried out at room temperature for 60 minutes and the determination of the residual plasmin at 37.5°C. It was found that the small air bubbles, entrapped in the fibrin clot, serve well as an indicator for the dissolution of the fibrin clot. By observation through a magnifying glass and under proper illumination, the time at which the majority of the rapidly released air bubbles reaches the meniscus of the reaction mixture was taken as the end point of the dissolution of the fibrin clot. Calculations of antifibrinolytic activity were essentially identical with those of Guest, Daly, Ware and Seegers(10,11).

Table II shows that fibrinolytic activity was demonstrable in the plasma of rats in "tourniquet shock," from 15 to 90 minutes after release of the tourniquets. The percentage of positive cases was comparatively low. In separate experiments with the "fibrinolysin" preparation mentioned previously, it was found that the amount of plasmin in the blood of rats in "tourniquet shock" was of the order of 0.009 to 0.018 fibrinolytic units in 1 ml of the 1:64 diluted plasma. In the blood of normal control rats, no fibrinolytic activity could be detected. Table II shows that antifibrinolytic activity in rat plasma gradually decreases during "tourniquet shock."

Since it is known that epinephrine is involved in the condition of stress, the effect of epinephrine administration on the fibrinolytic and antifibrinolytic titer was studied. Rats were injected intraperitoneally with 0.50 mg of epinephrine and the fibrinolytic and antifibrinolytic level of plasma determined after certain time intervals.

Fibrinolytic activity could be demonstrated shortly after injection of epinephrine (Table III). A maximum was reached after about 20 minutes, while after 45 minutes no activity was detectable. The actual plasmin values have been found to be, on the average, about

8. Loomis, E. C., and George, C., Jr., *Arch. Biochem.*, 1947, v12, 1.

9. MacFarlane, R. G., and Pilling, J., *Lancet*, 1946, v2, 562.

10. Guest, M. M., Ware, A. G., and Seegers, W. H., *Am. J. Physiol.*, 1947, v150, 661.

11. Guest, M. M., Daly, B. M., Ware, A. G., and Seegers, W. H., *J. Clin. Invest.*, 1948, v27, 785.

TABLE II. Fibrinolytic and Antifibrinolytic Activity in Plasma of Rats with Tourniquets on Both Hind Legs for 4½ Hours.

| Time after release of tourniquet, min. | Fibrinolytic activity: No. of animals used | Fibrinolytic activity: >.009 to .018 fibrinolytic units in 1 ml plasma (1:64) in: No. of animals | Fibrinolytic activity: >.009 to .018 fibrinolytic units in 1 ml plasma (1:64) in: % of animals | Antifibrinolytic activity: No. of animals used | Antifibrinolytic activity: Antifibrinolytic units per 1 ml of plasma (avg) |
|---|---|---|---|---|---|
| 0 | 9 | 0 | 0 | 5 | 164 |
| 15 | 7 | 1 | 14 | — | — |
| 30 | 23 | 3 | 13 | 4 | 156 |
| 45 | 37 | 9 | 24 | 15 | 153 |
| 60 | 24 | 4 | 17 | 4 | 146 |
| 90 | 10 | 2 | 20 | 14 | 126 |
| 120 | 6 | 0 | 0 | — | — |
| 180 | — | — | — | 12 | 131 |
| 300 | — | — | — | 8 | 139 |
| Control normal | 42 | 0 | 0 | 25 | 174 |

TABLE III. Fibrinolytic and Antifibrinolytic Activity in Plasma of Rats After Epinephrine Injection (0.50 mg).

| Time after epinephrine injection, min. | Fibrinolytic activity: No. of animals used | Fibrinolytic activity: >.018 to .036 fibrinolytic units in 1 ml plasma (1:64) in: No. of animals | Fibrinolytic activity: >.018 to .036 fibrinolytic units in 1 ml plasma (1:64) in: % of animals | Antifibrinolytic activity: No. of animals used | Antifibrinolytic activity: Antifibrinolytic units per 1 ml of plasma (avg) |
|---|---|---|---|---|---|
| 5 | 2 | 0 | 0 | — | — |
| 10 | 7 | 1 | 14 | — | — |
| 15 | 9 | 4 | 44 | 3 | 149 |
| 20 | 12 | 8 | 66 | 2 | 131 |
| 30 | 9 | 4 | 44 | 5 | 128 |
| 45 | 5 | 0 | 0 | 6 | 115 |
| 60 | 4 | 0 | 0 | 2 | 120 |
| 90 | 5 | 0 | 0 | 13 | 128 |
| 120 | 5 | 0 | 0 | 3 | 133 |
| 180 | — | — | — | 11 | 134 |
| 240 | 2 | 0 | 0 | 2 | 126 |
| 300 | — | — | — | 14 | 151 |
| 360 | 2 | 0 | 0 | — | — |
| 480 | — | — | — | 10 | 172 |
| Control normal | 42 | 0 | 0 | 25 | 174 |

twice as high as in the positively reacting blood of rats in "tourniquet shock," based on the dilution up to which fibrinolysis could be observed. The antifibrinolytic titer in plasma was decreased, reaching the lowest values after the disappearance of the fibrinolytic activity. The sharp initial decline, found 45 minutes after epinephrine administration, is followed by a slow rise of antifibrinolytic activity, reaching normal levels after about 8 hours. The similarity in the counterplay between plasmin and plasmin inhibitor in "tourniquet shock" and after epinephrine administration is apparent. The appearance and disappearance of fibrinolytic activity within a few minutes after epinephrine injection may suggest that plasmin is rapidly inactivated or eliminated or taken up by various tissues leading to an increased protein breakdown in these tissues.

Apparently, fibrinolytic activity could not be produced in rats as consistently as in humans(12). The dependability of demonstrating fibrinolytic activity may be limited due to a possible loss of plasmin from the plasma since it is strongly adsorbed by fibrin.

*Summary and conclusions.* No change in proteolytic activity could be demonstrated in the serum of rats in "tourniquet shock," using

12. Biggs, R., MacFarlane, R. G., and Pilling, J., *Lancet*, 1947, v1, 402.

the degree of liberation of tyrosine-tryptophane from a casein substrate as an index of proteolytic activity. The antitryptic titer in the same animals, measured on crystalline trypsin with casein as a substrate, was slightly lowered, compared to normal. Comparison of the fibrinogenolytic and proteolytic activities of trypsin, plasmin and the chloroform activated serum enzyme indicated parallelism between these enzymes. This similarity further substantiates the claim that plasmin can act as a proteolytic enzyme and may well play a significant role in the process of protein breakdown. It was found not to be feasible to use the destruction of the clotting ability of fibrinogen as a test method for fibrinolytic activity in blood of rats, subjected to "tourniquet shock." Fibrinolytic activity and reduced antifibrinolytic activity could be demonstrated in the plasma of rats in "tourniquet shock" and after epinephrine administration to normal rats. The fibrinolytic activity observed may be due either to an actual decrease in the concentration of the plasmin inhibitor or to an augmentation of the plasmin content. The recognition of plasmin and plasmin inhibitor as balancing components of a proteolytic system existing in normal blood greatly facilitates an approach to the problem of the mechanism of protein breakdown.

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